Aging induces endothelial dysfunction while sparing arterial thrombosis

Stämpfli, S F ; Akhmedov, A ; Gebhard, C ; Lohmann, C ; Holy, E W ; Rozenberg, I ; Spescha, R ; Shi, Y ; Lüscher, T F ; Tanner, F C ; Camici, G G

Abstract: OBJECTIVE: To assess the effects of aging on arterial thrombus formation by comparing 2-year-old with 11-week-old C57Bl6 mice. METHODS AND RESULTS: Aging is a major risk factor for cardiovascular disease. In humans, assessing the direct effects of aging on vascular homeostasis is difficult because it occurs in the presence of other risk factors. Arterial thrombosis is the critical event in cardiovascular diseases; however, it is not known whether aging per se promotes its occurrence. Mice represent an interesting system to address this issue because they age without spontaneously developing other risk factors. Organ chamber experiments confirmed the advanced level of aging of old mice. As previously shown, old mice exhibited endothelial dysfunction; however, arterial thrombosis induced by photochemical injury was unchanged. Arterial tissue factor expression and activity; expressions of tissue factor pathway inhibitor, thrombomodulin, and plasminogen activator inhibitor 1; prothrombin time; partial thromboplastin time; thrombin-antithrombin complex; and platelet activation were comparable in both groups. CONCLUSIONS: Although these results cannot be directly extrapolated to humans, this study contributes novel important information on the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals.

DOI: https://doi.org/10.1161/ATVBAHA.110.206920

Atherosclerosis, Thrombosis and Vascular Biology

Aging induces endothelial dysfunction while sparing arterial thrombosis

Simon F. Stämpfli[1,2,3], Alexander Akhmedov[1,2], Cathérine Gebhard[1,2,3], Christine Lohmann[1,2], Erik W. Holy[1,2,3], Izabela Rozenberg[1,2], Remo Spescha[1,2], Yi Shi [1,2], Thomas F. Lüscher[1,2,3], Felix C. Tanner[1,2,3], Giovanni G. Camici[1,2]

[1)] Cardiovascular Research, Institute of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland
[2)] Center for Integrative Human Physiology, University of Zurich
[3)] Cardiology, Cardiovascular Center, University Hospital Zurich

Running title: Aging, endothelial function, and arterial thrombosis

Word count of Body: 3808
Word count of abstract: 198
Number of figures: 5

Correspondence to:
Felix C. Tanner, MD
Cardiovascular Research, Institute of Physiology, University of Zurich
and Cardiology, Cardiovascular Center, University Hospital Zurich
Winterthurerstrasse 190, CH-8057 Zurich, Switzerland
felix.tanner@access.uzh.ch, Phone +41 44 635 64 69, Fax +41 44 635 68 27

Abstract

Objective: Aging is a major risk factor for cardiovascular disease. Assessing direct effects of aging on vascular homeostasis is difficult since in humans it occurs in the presence of other risk factors. Arterial thrombosis is the critical event in cardiovascular diseases; however, it is not known whether aging *per se* promotes its occurrence. Mice represent an interesting system to address this issue since they age without spontaneously developing other risk factors. Thus, we assessed the effects of aging on arterial thrombus formation by comparing 2 years old and 11 weeks old C57Bl6 mice.

Methods and Results: Organ chamber experiments confirmed the advanced level of aging of old mice. As previously shown, old mice exhibited endothelial dysfunction however arterial thrombosis induced by photochemical injury was unchanged. Arterial tissue factor expression and activity, expression of tissue factor pathway inhibitor, thrombomodulin, and plasminogen activator inhibitor 1 as well as prothrombin time, partial thromboplastin time, thrombin-antithrombin complex and platelet activation were comparable in both groups.

Conclusions: Although these results cannot be directly extrapolated to humans, this study contributes novel important information on the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals.

Key words: aging; endothelial function; atherosclerosis; cardiovascular risk factor; mouse

Condensed Abstract

Aging is a major risk factor for cardiovascular disease however its direct effect on acute arterial thrombosis is not known. Despite aging-dependent vascular dysfunction, thrombotic responses remain unchanged irrespective of the age. Although carried out on mice, this study sheds light of the pathogenesis of aging-induced cardiovascular disease.

Introduction

The percentage of elderly people in our society is steadily increasing and age is considered a major risk factor for the development of cardiovascular disease[1]. As most cardiovascular events such as myocardial infarction and ischemic stroke are caused by arterial thrombosis, it is tempting to speculate that advanced age promotes the development of arterial thrombosis. Yet, the direct effects of aging on arterial thrombosis have not been studied since aging in humans always occurs in parallel to other risk factors or atherosclerosis.

Unlike aged humans, old mice fed a regular diet do not spontaneously develop cardiovascular risk factors. Previous work showed that aged mice develop nitric oxide (NO) dependent endothelial dysfunction, but do not develop atherosclerotic lesions, diabetes or hypertension[2-5]. Therefore, mice represent an interesting system to study the process of aging in isolation and to investigate its direct effects on vascular homeostasis.

In light of the above, we compared 2 C57Bl6 male mice groups, bred and housed in same conditions, aged 2 years (old) and 11 weeks (young) and investigated age-induced changes in arterial thrombosis in the carotid artery. To this end, we employed an established photochemical injury model which principally affects the intimal layer as demonstrated by transmission electron microscopy (TEM) sections (data not shown) and mimics endothelial injury as occurring in atherothrombotic human complications[6, 7]. Endothelial function was assessed to document the stage of vascular aging. It was however not our intention to establish a causal link between NO-mediated endothelial dysfunction and age-related changes in thrombosis. In fact, this hypothesis had already been addressed by a previous study showing that mice lacking endothelial NO synthase (eNOS) exhibit no changes

in arterial thrombosis[8]. In light of the previous work linking inflammation and thrombosis[9] as well as inflammation an aging[10], the inflammatory state of young and old mice was assessed by immunhistochemistry and real time PCR.

Materials and Methods

Mice

Male C57BL/6 mice of the following age groups were examined: a) young group: 11 weeks, b) intermediate group: 62 weeks, and c) old group: 105 weeks. All mice were bred and housed in a specific pathogen free (SPF) animal facility with conventional light cycle at the Institute of Laboratory Animal Science, University of Zurich. All experiments were approved by the local authorities.

Organ chamber

Thoracic aorta was dissected free, excised, and immediately placed into ice-cold Krebs-Ringer bicarbonate solution. Vessels were cut into 3 mm rings and suspended in organ chambers for isometric tension recording (System 700 MO, Danish Myo Technology A/S, Aarhus, Denmark) containing 6 mL of Krebs solution (37°C, pH 7.4) aerated with 95% O_2/5% CO_2[11]. After a 30 min equilibration period, rings were progressively stretched to the optimal point of their length-tension curve, and the presence of a functional endothelium was tested in norepinephrine precontracted rings by adding 10^{-7} mol/L acetylcholine. Endothelial function was assessed in norepinephrine precontracted rings by studying the relaxation to acetylcholine (10^{-9} to 10^{-4} mol/L; Sigma–Aldrich) in the presence or absence of L-NAME (3×10^{-4} mol/L; 30 min incubation, Sigma–Aldrich). The NO donor sodium nitroprusside (10^{-9} to 10^{-4} mol/L; Sigma–Aldrich) was applied to test endothelium-independent relaxations. Concentration-dependent contractions were established by norepinephrine (10^{-9} to 10^{-4} mol/L; Sigma–Aldrich).

Arterial thrombosis

Mice were anesthetized with pentobarbital (87 mg/kg i.p.). Rose bengal (Fisher Scientific, Fair Lawn, NJ) was injected into the tail vein in a volume of 0.12 mL at a concentration of 50 mg/kg body weight. Mice were placed under a dissecting microscope and the right common carotid artery was exposed following a midline cervical incision. A Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) was applied and connected to a flowmeter (Transonic, Model T106) supplying a data acquisition system (PowerLab 4/30, AD Instruments). A 1.5-mW green light laser (540 nm) (Melles Griot, Carlsbad, CA) was directed at the desired site of injury at a distance of 6 cm for 60 minutes or until complete occlusion. Flow was monitored for 120 minutes from the onset of injury. Occlusion was defined as flow ≤0.1 mL/min for at least 1 minute[7]. Data were analyzed with ChartPro Software (AD Instruments). The photochemical injury protocol leads to an endothelial-specific injury largely confined to the diameter of the laser beam which reacts with the laser-sensitive rose bengal. As a result of the photochemical injury, endothelial and subendothelial procoagulant factors come in contact with circulating coagulation factors resulting in the formation of an arterial thrombus. Kinetics of and time to occlusion are monitored in real-time with a Doppler probe.

Tissue factor activity

Carotid arteries were homogenized in 50 µl of lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1% Triton-X 100, pH 7.4) by manual grinding on ice. Samples were then centrifuged at 14000 rpm for 15 min at 4°C and the supernatant was transferred to a fresh tube. Tissue factor (TF) activity was assessed with a colorimetric assay (Actichrome TF, American Diagnostica); incubation at 37°C allowed TF to form a complex with FVII and this complex to cleave FX to FXa. Absorbance was

determined at 405 nm and compared to a standard curve generated using known amounts of lyophilized TF.

Coagulation pathways

Plasma from citrated blood (3.2% citrate 1/10) was extracted by 15 minutes centrifugation (2500g, 4°C) and stored immediately at -80°C until analysis. Prothrombin time (PT) and partial thromboplastin time (aPTT) were assessed by the Start4 analyzer (Diagnostica Stago, France), using the according reagents (Roche Diagnostics, Switzerland). Thrombin-antithrombin (TAT) complex was measured by sandwich ELISA (Enzyme Research Laboratories, USA).

Shear stress-dependent platelet function

Shear stress-dependent platelet function was assessed with a cone and platelet analyzer. Citrated whole blood (200 µL) was circulated in polystyrene wells at a shear rate of 1875 s^{-1} for 2 minutes with a rotating polytetrafluoroethylene cone as described[12-14]. Wells were washed, stained with May-Grünwald dye, and analyzed with a microscope connected to an image analysis system (ImageJ). Results are expressed as the percentage of surface covered by platelets.

Real-time PCR

Aortic arches were snap frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was generated using Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscience) and first-strand cDNA primer $pd(N)_6$. Real-time PCR was performed using SybrGreen Jump start kit (Sigma). Murine primers were as follows: tissue factor (TF): forward (5'-3') CAATGAATTCTCGATTGATGTGG, reverse: (5'-3')

GGAGGATGATAAAGATGGTGGC; tissue factor pathway inhibitor (TFPI): forward (5'-3') ACTGTGTGTCTGTTGCTTAGCC, reverse: (5'-3') GTTCTCGTTCCCTTCACATCCC; plasminogen activator inhibitor (PAI-1): forward (5'-3') ATCAATGACTGGGTGGAAAGGC, reverse: (5'-3') GTTGAACTTGTTGCTCTGAGCCthrombomodulin (TM): forward (5'-3') TTTCTTTCTGGGTGTGCTGGC, reverse: (5'-3') TGCTCTGGCTCAGCAGAAGG; matrix metalloproteinase 9 (MMP-9): forward (5'-3') CCTGGAACTCACACGACATCTTC, reverse: (5'-3') TGGAAACTCACACGCCAGAA; eNOS: forward (5'-3') ATTAAATACGCAACAAATAGAGGC, reverse: (5'-3') CTCAGTGATCTCCACGTTGGC. Data were normalized to murine S12: forward (5'-3') GAAGCTGCCAAAGCCTTAGA, reverse (5'-3') AACTGCAACCAAAACCCTTC. The amplification program consisted of 1 cycle at 95˚C for 10 min, followed by 40 cycles with a denaturing phase at 95˚C for 30 s, an annealing phase of 1 min at 60˚C and an elongation phase at 72˚C for 1 min. For verification of the correct amplification, PCR products were analyzed on an ethidium bromide stained 1,5% agarose gel. Quantification was performed using $2^{-\Delta\Delta Ct}$ method.

Blood glucose, total cholesterol, plasma triglycerides

All measurements were performed in the morning after a 14 hours fasting period. Glucose was measured in whole blood with ACCU-CHEK aviva system (Roche). Cholesterol and triglycerides were determined in heparinized plasma (15 minutes centrifugation, 2500g, 4°C) by the enzymatic CHOD-PAP and GPO-PAP method (both from Roche), respectively.

Immunohistochemistry

Aortic roots and carotid arteries were mounted in O.C.T. (Tissue-Tek).

Specimens were cut into 8 mm sections, fixed with 4% paraformaldehyde. Sections were stained with Oil Red-O (ORO) for 1 hour at room temperature. For the assessment of myeloperoxidase (MPO; Labvision), CD68 (Serotec), and tumor necrosis factor a (TNF-α; Abcam) sections were fixed in acetone, blocked with 1% FCS, and incubated with specific primary antibody. Primary antibodies were revealed by goat anti-species specific immunoglobulin antibodies, followed by alkaline phosphatase-labelled donkey anti-goat antibodies. Alkaline phosphatase was visualized using naphtol AS-BI phosphate and new fuchsin as substrate. Sections were counterstained with hemalaun and coverslipped

Statistics

Data are represented as mean±SEM. Statistical analysis was performed by 2-tailed unpaired Student *t* test or ANOVA as appropriate. A probability value of <0.05 was considered significant.

Results

Vascular tone

Endothelium-dependent relaxation to acetylcholine was impaired in old as compared to young mice (maximal relaxation: -58.9±9.6% vs. -88.0±4.4%, $p<0.01$; -log ED50: 6.52±0.27 vs. 7.05±0.09, p=n.s.; n=5; Figure 1A). The nitric oxide synthase inhibitor L-NAME abrogated acetylcholine induced relaxation in both groups (maximal response: 8.6±5.2% vs. 0.08±3.5%; p=n.s.; n=5; Figure 1A). In contrast, endothelium-independent relaxation to sodium nitroprusside was similar in old and young mice (maximal relaxation: -93.3±6.8% vs. -111.9±7.4%, p=n.s.; -log ED50: 7.89±0.21 vs. 7.69±0.17, p=n.s.; n=5; Figure 1B). No difference was observed in the contraction to KCl (0.79±0.07 g vs. 0.71±0.07 g; p=n.s.; n=10; Figure 1C), L-NAME (11.1±10.9% vs. 10.4±13.2%; p=n.s.; n=5; Figure 1D), or norepinephrine (118.7±16.3% vs. 138.9±13.8%; p=n.s.; n=5; Figure 1E). Levels of eNOS mRNA expression were comparable in young and old mice (p=0.0739; n=5; data not shown).

Arterial thrombosis

Age-related changes in arterial thrombosis were examined by a carotid artery photochemical injury model and assessed by real time blood flow measurement. Both time to thrombotic occlusion and the kinetics of thrombus formation were unaltered in old (45.5±3.7 min; n=5) as compared to middle aged (53.0±5.8 min; p=n.s.; n=6;) or young mice (45.6±5.9 min; p=n.s.; n=5; Figures 2A and 2B). Carotid artery blood flow before initiation of photochemical injury was similar in old and young mice (0.81±0.07 vs. 0.77±0.07 mL/min; p=n.s.; n=5).

To improve our understanding of the laser-induced damage to the arterial wall, we

examined sections of non-injured and injured carotid arteries by electron microscopy. Both the internal elastic membrane and the vascular media remained morphologically intact after the laser injury under our experimental conditions indicating that the injury is largely confined to the endothelial layer (data not shown).

Analysis of coagulatory and fibrinolytic factors and platelets

TF activity in carotid artery did not differ between old and young mice (OD405: 0.68±0.01 vs. 0.5±0.05; p=n.s.; n=5; Figure 3A). In line with this, TF mRNA levels ($\Delta\Delta C_t$: 3.7±1.1 vs. 4.8±1.5; p=n.s.; n=5; Figure 3A) as well as levels of its antagonist TFPI ($\Delta\Delta C_t$: 0.0134±0.0028 vs. 0.0120±0.0017; p=n.s.; n=5; Figure 3B) were comparable in old and young mice. PAI-1 ($\Delta\Delta C_t$: 0.210±0.049 vs. 0.408±0.088; p=0.0862; n=5; Figure 3B) as well as TM expression ($\Delta\Delta C_t$: 0.342±0.087 vs. 0.434±0.153; p=n.s.; n=5; Figure 3B) were not different between the two groups. Extrinsic and intrinsic coagulation pathways were examined by measuring PT and aPTT, respectively. No changes were observed between old and young mice neither for PT (10.7±0.23 vs. 10.9±0.25 s; p=n.s.; n=5; Figure 3C) nor aPTT (26.7±2.8 vs. 26.2±1.5 s; p=n.s.; n=5; Figure 3C). TAT complex was assayed in plasma and no difference was found between young and old mice (4.79±0.16 vs. 4.47±0.093 ng/mL; p=0.1236; N=5; data not shown). Platelet function was assessed by determining shear stress-dependent platelet adhesion. Old and young mice exhibited similar values (5.7±2.0% vs. 4.1±1.5%; p=n.s.; n=5; Figure 3D).

Arterial morphology

Aortic roots, descending aortae, and carotid arteries were stained by HE and Oil-Red-O. Histological features of the arteries were comparable in old and young mice (Figure 4). No lipid deposits or fatty streaks were detected in either group. In the

aortic root, sporadic single subendothelial cells stained positive for Oil-Red-O in old, but not in young mice (Figure 4B).

Blood glucose, plasma cholesterol, and plasma triglycerides

Fasting blood glucose (81.1±8.3 vs. 60.8±1.3 mg/dL; p=n.s) and total plasma cholesterol (81.2±2.2 vs. 59.3±20.3 mg/dL; p=n.s) did not differ in old and young mice. Plasma triglyceride levels after 14 hours of fasting were significantly lower in old mice as compared to young controls (35.6±7.9 vs. 99.8±4.2 mg/dL; $p<0.001$).

MMP-9 expression and immunohistochemical analysis of inflammation

Levels of MMP-9 mRNA expression were comparable in both groups. ($\Delta\Delta C_t$: 0.00023±0.00004 vs. 0.00026±0.00006; p=n.s.; n=5, Figure 5). Immunohistochemical analysis of carotid arteries revealed comparable expression levels of the inflammatory markers MPO, CD68, and TNF-α in young and old mice indicating a similar and very low level of inflammation in both groups (Figure 5).

Discussion

Increasing age is considered a major risk factor for the development of cardiovascular disease[1]. Thus, studying its molecular mechanisms is an important scientific goal especially in view of the growing percentage of elderly people in our society. This study demonstrates that aging *per se* does not promote arterial thrombosis. Endothelial function was assessed in order to document the advanced stage of vascular aging of old mice but not to establish a direct link between NO-mediated endothelial function and thrombosis. This link had indeed been investigated in a previous study were deletion of eNOS gene was shown not to affect arterial thrombus formation[8]. Old mice indeed exhibited impaired endothelium-dependent relaxation, while maintaining an unaltered thrombotic potential. Moreover, the kinetics of thrombus formation, arterial TF activity, plasma coagulation times, and platelet adhesion as well as modulators of coagulation (TFPI, PAI-1, TM) remained unchanged as compared to young controls. Although levels of PAI-1 and eNOS were not statistically different, a trend was visible and we cannot fully exclude that a higher number of animals might have yielded a different result. Mice have a life expectancy of roughly two years; and the aged mice used in this study were over one hundred and four weeks old; thus, their age corresponded roughly to seventy/eighty years of age for a human.

Mice fed a regular diet do not exhibit elevated blood pressure, glucose, or cholesterol with age[15]. Indeed, not only remained fasting blood glucose and plasma cholesterol levels unaltered in old mice, fasting triglyceride levels were even decreased in these animals; hence, in line with previous studies[3], old mice fed their entire lifespan a normocaloric diet did not exhibit glucose, cholesterol, or triglyceride levels known to be associated with vascular disease. Consistent with these observations, histological analysis did not reveal atherosclerotic lesions such as fatty

streaks in aortic roots, carotid arteries, or descending aortas of old mice. Sporadic single cells stained positive for Oil Red-O in the aortic root, one of the typical sites of atherogenesis in mice[16], most likely representing intracellular lipids; nevertheless, accumulation of inflammatory cells or extracellular lipids were not noted. Vascular inflammation, assessed by immuohistochemical staining for the inflammatory marker MPO, macrophage marker CD68, and the inflammatory cytokine TNF-α as well as expression of MMP-9 was very low in both groups.

Rupture of atherosclerotic plaques and consequent thrombus formation is the main cause of acute cardiovascular events in humans. A very important question in this context is whether the increasing incidence of thrombosis with aging solely depends on more severe atherosclerosis or whether aging promotes thrombosis by itself. In light of these considerations, the absence of atherosclerosis in our mouse model is a prerequisite for this study. Hence, we were especially interested in studying aging-related changes in the absence of atherosclerosis in order to dissect these two parameters. In light of the above and despite the obvious differences to humans, mice represent an interesting model for the independent assessment of aging on vascular function or, like in the present study, for investigating the direct effect of aging on arterial thrombus formation.

Following the photochemical injury protocol, old mice displayed unaltered time to thrombotic occlusion compared to young ones. Furthermore, the kinetics of arterial thrombus formation, as assessed by measuring real time blood flow following photochemical injury, was comparable. Both the internal elastic membrane and the vascular media remained morphologically intact after the laser injury under our experimental conditions, as confirmed by TEM (data not shown). Altogether, the model has many similarities with an endothelial erosion, which is a well known entity triggering an occlusive arterial thrombosis in humans. Hence, these data provide

convincing evidence that aging per se does not affect arterial thrombosis in the mouse in the absence of additional cardiovascular risk factors or atherosclerosis.

Age-related endothelial dysfunction occurs in both human[17] and murine[3] arteries and is characterized by a decreased bioavailability of NO[2, 18, 19]. Since aging and other risk factors[20] occur together in humans, their independent assessment is extremely difficult. The old mice examined in this study, exhibited a markedly dysfunctional endothelium but no additional risk factor or atherosclerosis; moreover, the decreased endothelium-dependent relaxation in old mice underscores a relevant impairment of vascular function, providing appropriate conditions to study the impact of age-related vascular alterations on arterial thrombosis. These alterations are restricted to the endothelium, since endothelium-independent relaxations to sodium nitroprusside as well as contractions to both receptor-dependent and -independent agonists remained unaltered. Moreover, the observed alterations are solely functional, since histological analysis of the arteries did not reveal any structural difference between old and young mice. Furthermore, levels of eNOS mRNA expression were comparable as also shown in a previous study[3]. Furthermore, genetic deletion of eNOS does not influence arterial thrombus formation[8]. In line with this, despite endothelial dysfunction in the old mice, arterial thrombosis in these animals remained unaltered as compared to young controls, indicating that aging *per se* does not directly affect arterial thrombosis.

In both humans and mice, age-related changes of coagulation factors have been reported. Factor VIII, factor VII, and fibrinogen plasma levels increase with age in humans[21-23] and mice[23]. In the former, some of these alterations are associated with coronary artery disease or overall mortality, although no causal relationship has been established[24]. Such prothrombotic alterations may well be counterbalanced in humans by increased plasma levels of anticoagulant proteins like TFPI or protein C,

which have been reported as well[25]. Moreover, such changes may occur secondary to activation of coagulation by endothelial damage or endothelial erosion[26] rather than playing a direct role in thrombosis. In addition, many of these parameters are acute phase reactants and may simply represent an enhanced inflammatory burden[27]. Hence, the clinical relevance of such changes in plasma coagulation factors with aging is questionable. Consistent with this interpretation, studies in centenarians revealed that these individuals exhibit higher levels of procoagulant factors than old controls, suggesting that such changes correlate with health and longevity and do not necessarily lead to a higher risk for arterial thrombosis. In addition, mRNA expression and protein activity of TF, the main trigger of coagulation[28], and expression of its antagonist TFPI as well as PAI-1 and TM were not altered in these animals. PT, aPTT and TAT complex are globally accepted parameters for assessing changes in coagulation[29]; the integral character of these tests renders them well suited for assessing the activity of coagulation pathways. Age-related changes of these parameters have not been described[30], and clinically used reference ranges are not age-dependent[31]. In line with these observations and with the reported similar kinetics of arterial thrombosis in vivo, PT, aPTT and TAT complex remained unchanged in old mice. Therefore, no functionally relevant alteration of the coagulation system was apparent. Additionally, given the unchanged thrombotic potential and unchanged PAI-1 levels observed in young and old mice it is conceivable to expect that also activation of the fibrinolytic system did not vary with age.

Platelets are crucially involved in arterial thrombus formation. In aged humans – not controlled for classical risk factors – elevated levels of β-thromboglobulin and platelet factor 4[32] as well as a lower aggregation threshold to adenosine diphosphate and collagen[33] have been reported. In addition, von Willebrand factor expression is

known to increase with age and correlate with age related risk factors[34] and may in these subjects enhance platelet activation in vivo. Furthermore, phospholipid composition of platelets differs with age in healthy subjects[35], suggesting an age related alteration in transmembrane signaling. In the present study, platelet function was assessed by shear stress induced adherence. No age-dependent alteration in platelet function was recorded in these experiments suggesting comparable platelet function among young and old mice, implying that the age-related changes described may not be directly influenced by aging but rather by other risk factors.

In summary, this study shows that aging *per se* does not affect arterial thrombosis in the mouse and that age-dependent vascular dysfunction in the absence of additional risk factors does not alter arterial thrombus formation. Although limited by the use of mice, this study contributes novel important information concerning the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals. Studying the effect of additional risk factors and/or atherosclerosis in concert with aging on arterial thrombosis could certainly represent an interesting future development of this study.

Sources of Funding

The study was supported by Swiss National Science Foundation (to TFL, FCT and GGC), Velux Foundation, Wolfermann Nägeli Foundation, MERCATOR foundation, Hartmann Müller-Stiftung and Olga Mayenfish fountadion (to GGC) and the Swiss Heart Foundation.

Disclosures

None.

References

1. Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell C, Roger V, Sorlie P, Steinberger J, Thom T, Wilson M, Hong Y. Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation.* 2008;117:e25-146.
2. Tschudi MR, Barton M, Bersinger NA, Moreau P, Cosentino F, Noll G, Malinski T, Luscher TF. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. *J Clin Invest.* 1996;98:899-905.
3. Francia P, delli Gatti C, Bachschmid M, Martin-Padura I, Savoia C, Migliaccio E, Pelicci PG, Schiavoni M, Luscher TF, Volpe M, Cosentino F. Deletion of p66shc gene protects against age-related endothelial dysfunction. *Circulation.* 2004;110:2889-2895.
4. Tiemann K, Weyer D, Djoufack PC, Ghanem A, Lewalter T, Dreiner U, Meyer R, Grohe C, Fink KB. Increasing myocardial contraction and blood pressure in C57BL/6 mice during early postnatal development. *Am J Physiol Heart Circ Physiol.* 2003;284:H464-474.
5. Thomas MK, Devon ON, Lee JH, Peter A, Schlosser DA, Tenser MS, Habener JF. Development of diabetes mellitus in aging transgenic mice following suppression of pancreatic homeoprotein IDX-1. *J Clin Invest.* 2001;108:319-329.
6. Camici GG, Steffel J, Amanovic I, Breitenstein A, Baldinger J, Keller S, Luscher TF, Tanner FC. Rapamycin promotes arterial thrombosis in vivo: implications for everolimus and zotarolimus eluting stents. *Eur Heart J.* 2009.

7. Camici GG, Steffel J, Akhmedov A, Schafer N, Baldinger J, Schulz U, Shojaati K, Matter CM, Yang Z, Luscher TF, Tanner FC. Dimethyl sulfoxide inhibits tissue factor expression, thrombus formation, and vascular smooth muscle cell activation: a potential treatment strategy for drug-eluting stents. *Circulation.* 2006;114:1512-1521.
8. Ozuyaman B, Godecke A, Kusters S, Kirchhoff E, Scharf RE, Schrader J. Endothelial nitric oxide synthase plays a minor role in inhibition of arterial thrombus formation. *Thromb Haemost.* 2005;93:1161-1167.
9. Willerson JT, Ridker PM. Inflammation as a cardiovascular risk factor. *Circulation.* 2004;109:II2-10.
10. Chung HY, Sung B, Jung KJ, Zou Y, Yu BP. The molecular inflammatory process in aging. *Antioxid Redox Signal.* 2006;8:572-581.
11. Camici GG, Schiavoni M, Francia P, Bachschmid M, Martin-Padura I, Hersberger M, Tanner FC, Pelicci P, Volpe M, Anversa P, Luscher TF, Cosentino F. Genetic deletion of p66(Shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. *Proc Natl Acad Sci U S A.* 2007;104:5217-5222.
12. Flammer AJ, Hermann F, Sudano I, Spieker L, Hermann M, Cooper KA, Serafini M, Luscher TF, Ruschitzka F, Noll G, Corti R. Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation.* 2007;116:2376-2382.
13. Hermann F, Spieker LE, Ruschitzka F, Sudano I, Hermann M, Binggeli C, Luscher TF, Riesen W, Noll G, Corti R. Dark chocolate improves endothelial and platelet function. *Heart.* 2006;92:119-120.
14. Shenkman B, Savion N, Dardik R, Tamarin I, Varon D. Testing of platelet deposition on polystyrene surface under flow conditions by the cone and

plate(let) analyzer: role of platelet activation, fibrinogen and von Willebrand factor. *Thromb Res.* 2000;99:353-361.

15. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis.* 1987;68:231-240.
16. Nakashima Y, Plump AS, Raines EW, Breslow JL, Ross R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler Thromb.* 1994;14:133-140.
17. Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol.* 1994;24:471-476.
18. Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, Salvetti A. Age-related reduction of NO availability and oxidative stress in humans. *Hypertension.* 2001;38:274-279.
19. van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, Palacios-Callender M, Erusalimsky JD, Quaschning T, Malinski T, Gygi D, Ullrich V, Luscher TF. Enhanced peroxynitrite formation is associated with vascular aging. *J Exp Med.* 2000;192:1731-1744.
20. Celermajer DS, Sorensen KE, Bull C, Robinson J, Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol.* 1994;24:1468-1474.
21. Tracy RP, Arnold AM, Ettinger W, Fried L, Meilahn E, Savage P. The relationship of fibrinogen and factors VII and VIII to incident cardiovascular

disease and death in the elderly: results from the cardiovascular health study. *Arterioscler Thromb Vasc Biol.* 1999;19:1776-1783.

22. Tofler GH, Massaro J, Levy D, Mittleman M, Sutherland P, Lipinska I, Muller JE, D'Agostino RB. Relation of the prothrombotic state to increasing age (from the Framingham Offspring Study). *Am J Cardiol.* 2005;96:1280-1283.
23. Gulledge AA, Rezaee F, Verheijen JH, Lord ST. A novel transgenic mouse model of hyperfibrinogenemia. *Thromb Haemost.* 2001;86:511-516.
24. Danesh J, Lewington S, Thompson SG, Lowe GD, Collins R, Kostis JB, Wilson AC, Folsom AR, Wu K, Benderly M, Goldbourt U, Willeit J, Kiechl S, Yarnell JW, Sweetnam PM, Elwood PC, Cushman M, Psaty BM, Tracy RP, Tybjaerg-Hansen A, Haverkate F, de Maat MP, Fowkes FG, Lee AJ, Smith FB, Salomaa V, Harald K, Rasi R, Vahtera E, Jousilahti P, Pekkanen J, D'Agostino R, Kannel WB, Wilson PW, Tofler G, Arocha-Pinango CL, Rodriguez-Larralde A, Nagy E, Mijares M, Espinosa R, Rodriquez-Roa E, Ryder E, Diez-Ewald MP, Campos G, Fernandez V, Torres E, Marchioli R, Valagussa F, Rosengren A, Wilhelmsen L, Lappas G, Eriksson H, Cremer P, Nagel D, Curb JD, Rodriguez B, Yano K, Salonen JT, Nyyssonen K, Tuomainen TP, Hedblad B, Lind P, Loewel H, Koenig W, Meade TW, Cooper JA, De Stavola B, Knottenbelt C, Miller GJ, Cooper JA, Bauer KA, Rosenberg RD, Sato S, Kitamura A, Naito Y, Palosuo T, Ducimetiere P, Amouyel P, Arveiler D, Evans AE, Ferrieres J, Juhan-Vague I, Bingham A, Schulte H, Assmann G, Cantin B, Lamarche B, Despres JP, Dagenais GR, Tunstall-Pedoe H, Woodward M, Ben-Shlomo Y, Davey Smith G, Palmieri V, Yeh JL, Rudnicka A, Ridker P, Rodeghiero F, Tosetto A, Shepherd J, Ford I, Robertson M, Brunner E, Shipley M, Feskens EJ, Kromhout D, Dickinson A, Ireland B, Juzwishin K, Kaptoge S, Lewington S, Memon A, Sarwar N, Walker M, Wheeler J, White I, Wood A. Plasma fibrinogen level and

the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *Jama.* 2005;294:1799-1809.

25. Sakkinen PA, Cushman M, Psaty BM, Kuller LH, Bajaj SP, Sabharwal AK, Boineau R, Macy E, Tracy RP. Correlates of antithrombin, protein C, protein S, and TFPI in a healthy elderly cohort. *Thromb Haemost.* 1998;80:134-139.
26. Mari D, Coppola R, Provenzano R. Hemostasis factors and aging. *Exp Gerontol.* 2008;43:66-73.
27. Tracy RP. Hemostatic and inflammatory markers as risk factors for coronary disease in the elderly. *Am J Geriatr Cardiol.* 2002;11:93-100, 107.
28. Steffel J, Luscher TF, Tanner FC. Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. *Circulation.* 2006;113:722-731.
29. Bajaj SP, Joist JH. New insights into how blood clots: implications for the use of APTT and PT as coagulation screening tests and in monitoring of anticoagulant therapy. *Semin Thromb Hemost.* 1999;25:407-418.
30. Lewandowski K, Kulinska A, Zozulinska M, Zawilska K. Age-related changes in plasma endogenous heparin-like activity in healthy male volunteers. *Thromb Res.* 1991;62:109-114.
31. Kamal AH, Tefferi A, Pruthi RK. How to interpret and pursue an abnormal prothrombin time, activated partial thromboplastin time, and bleeding time in adults. *Mayo Clin Proc.* 2007;82:864-873.
32. Zahavi J, Jones NA, Leyton J, Dubiel M, Kakkar VV. Enhanced in vivo platelet "release reaction" in old healthy individuals. *Thromb Res.* 1980;17:329-336.
33. Kasjanovova D, Balaz V. Age-related changes in human platelet function in vitro. *Mech Ageing Dev.* 1986;37:175-182.
34. Conlan MG, Folsom AR, Finch A, Davis CE, Sorlie P, Marcucci G, Wu KK. Associations of factor VIII and von Willebrand factor with age, race, sex, and

risk factors for atherosclerosis. The Atherosclerosis Risk in Communities (ARIC) Study. *Thromb Haemost.* 1993;70:380-385.

35. Prisco D, Rogasi PG, Matucci M, Paniccia R, Abbate R, Gensini GF, Serneri GG. Age related changes in platelet lipid composition. *Thromb Res.* 1986;44:427-437.

Figure legends

Figure 1

Isometric tension studies in aortic rings of old (filled symbols) and young (open symbols) mice **A:** Endothelium-dependent relaxations to acetylcholine are impaired in old as compared to young mice (*p<0.01; **p<0.001; n=5). Pretreatment with L-NAME abrogates relaxations in both groups (p=n.s. for old vs. young mice; p<0.001 for control vs. L-NAME; n=5). **B:** Endothelium-independent relaxations to sodium nitroprusside do not differ between old and young mice (p=n.s.; n=5). **C:** Contractions to KCl (80 mM) are similar in old and young mice (p=n.s.; w=weeks; n=5). **D:** Contractions to L-NAME ($3x10^{-4}$ mol/L) are similar in old and young mice (p=n.s.; w=weeks; n=5). **E:** Contractions to norepinephrine are similar in old and young mice (p=n.s.; n=5).

Figure 2

Aging does not affect arterial thrombus formation **A:** Old and young mice exhibit similar time to thrombotic occlusion (p=n.s.; n=5). **B:** Real time blood flow plot analysis reveals no difference in the flow pattern over time (representative records).

Figure 3

TF expression and activity, expression of TFPI, PAI-1, and TM, coagulation times, and platelet adhesion in old and young mice **A:** Old and young mice exhibit comparable arterial TF activity and TF mRNA (p=n.s.; n=5). **B:** No significant difference in mRNA expression of TFPI, PAI-1 and TM was observed. **C:** Prothrombin time (PT) and partial thromboplastin time (aPTT) do not differ in old and young mice (p=n.s.; n=5). **D:** Shear-stress dependent platelet adhesion is similar in old and young mice (p=n.s.; n=5).

Figure 4

Similar arterial histology in old and young mice **A:** No structural difference is evident in aortic roots of old and young mice (HE stain). **B:** No lipid deposits are detected in aortic roots of old and young mice, while sporadic single subendothelial Oil-red-O positive cells are detectable in old mice. Descending aorta **(C)** and carotid arteries **(D)** exhibit similar histology in old and young mice.

Figure 5

Expression of inflammatory markers MPO, CD-68, and TNF-α in the arterial wall **A:** Expression of the neutrophil marker MPO was comparable in old and young mice. **B:** Macrophage marker CD68 was expressed to a similar extent in both groups. **C:** The pro-inflammatory cytokine TNF-α exhibited a similar expression pattern in old and young mice. **D:** mRNA levels of MMP-9, assessed by real time PCR analysis, were comparable in both groups.

Figure 1

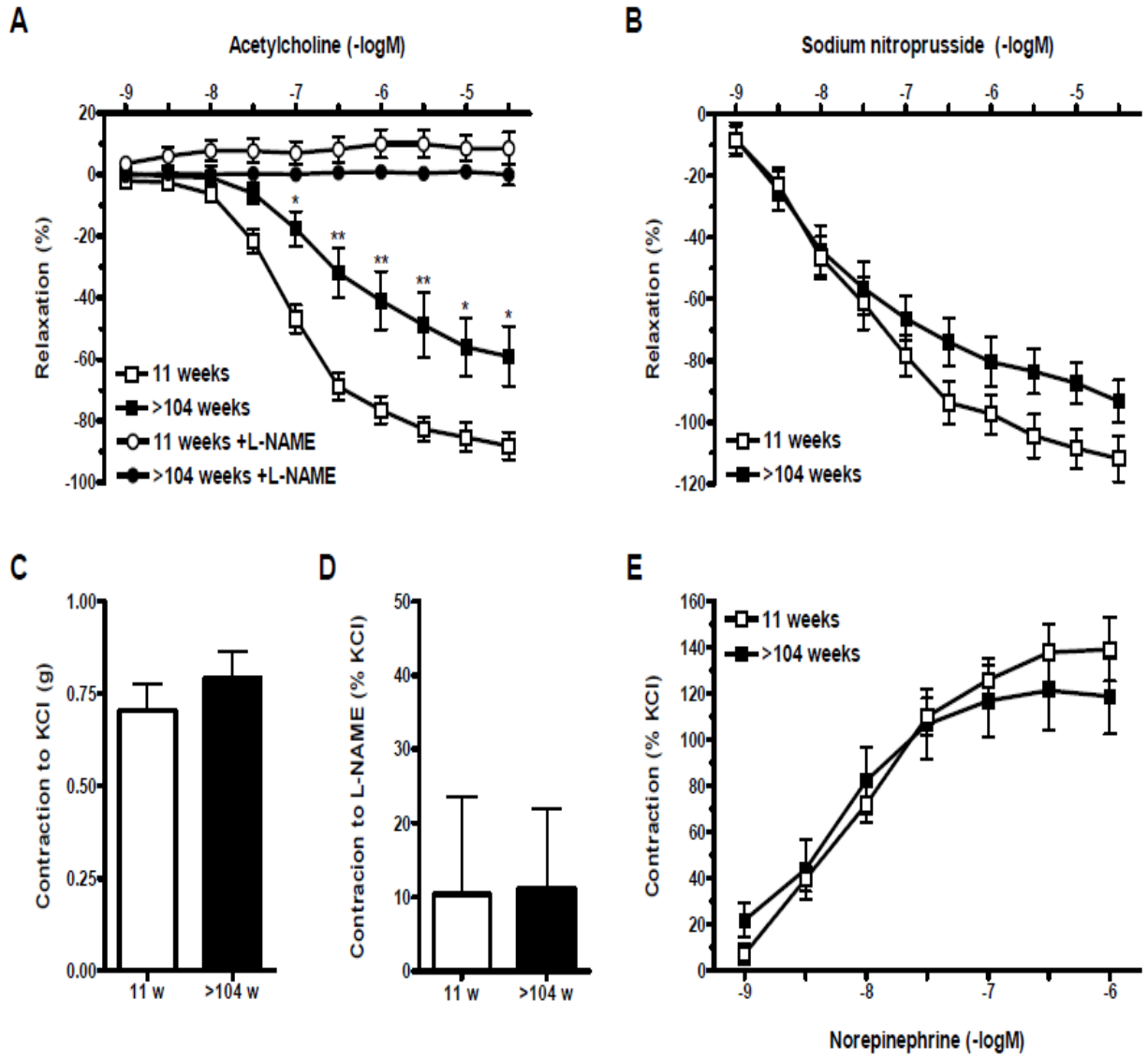

A
Acetylcholine (-logM)
Relaxation (%)
11 weeks
>104 weeks
11 weeks +L-NAME
>104 weeks +L-NAME
B
Sodium nitroprusside (-logM)
Relaxation (%)
11 weeks
>104 weeks
C
Contraction to KCl (g)
11 w
>104 w
D
Contracion to L-NAME (% KCl)
11 w
>104 w
E
Contraction (% KCl)
11 weeks
>104 weeks
Norepinephrine (-logM)

Figure 2

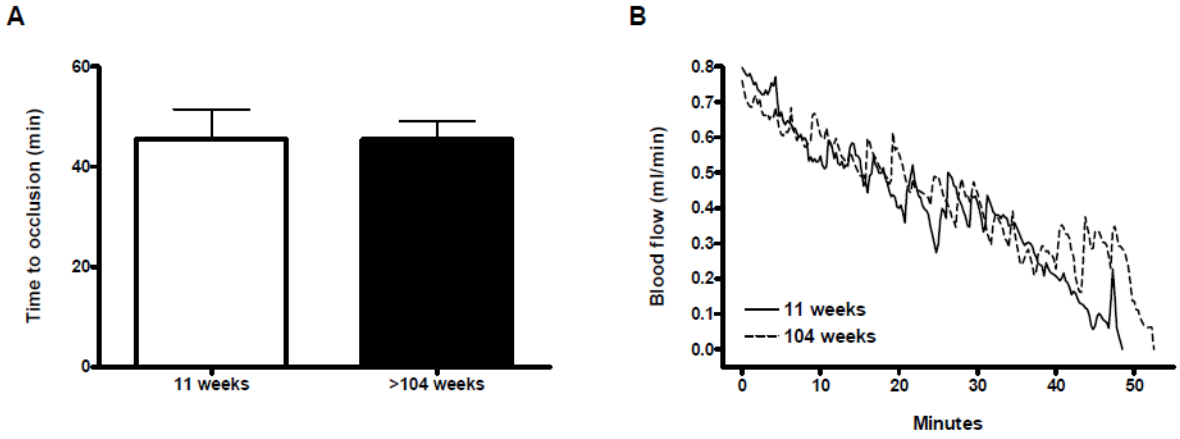
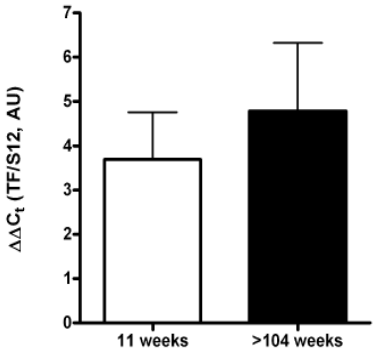
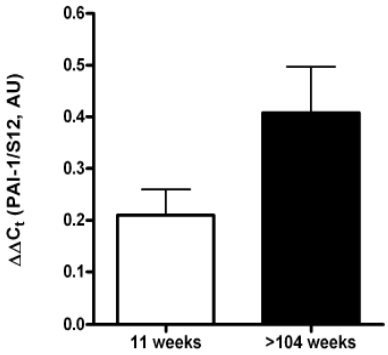
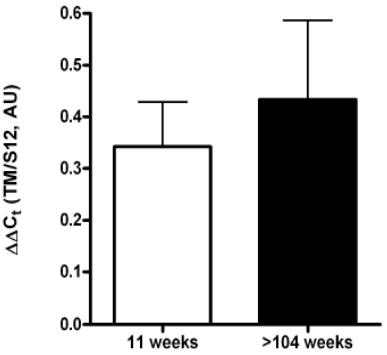
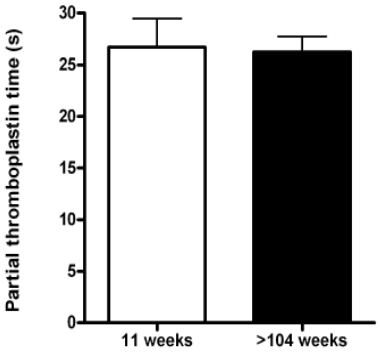

A
Time to occlusion (min)
60
40
20
0
11 weeks
>104 weeks
B
Blood flow (ml/min)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
11 weeks
104 weeks
0
10
20
30
40
50
Minutes

Figure 3


ΔΔCt (TF/S12, AU)
0
1
2
3
4
5
6
7
11 weeks
>104 weeks


ΔΔCt (PAI-1/S12, AU)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
11 weeks
>104 weeks


ΔΔCt (TM/S12, AU)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
11 weeks
>104 weeks


Partial thromboplastin time (s)
0
5
10
15
20
25
30
11 weeks
>104 weeks

D

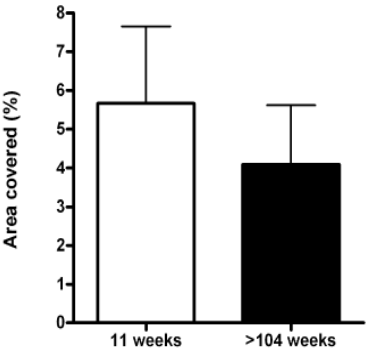

Area covered (%)
0
1
2
3
4
5
6
7
8
11 weeks
>104 weeks

Figure 4

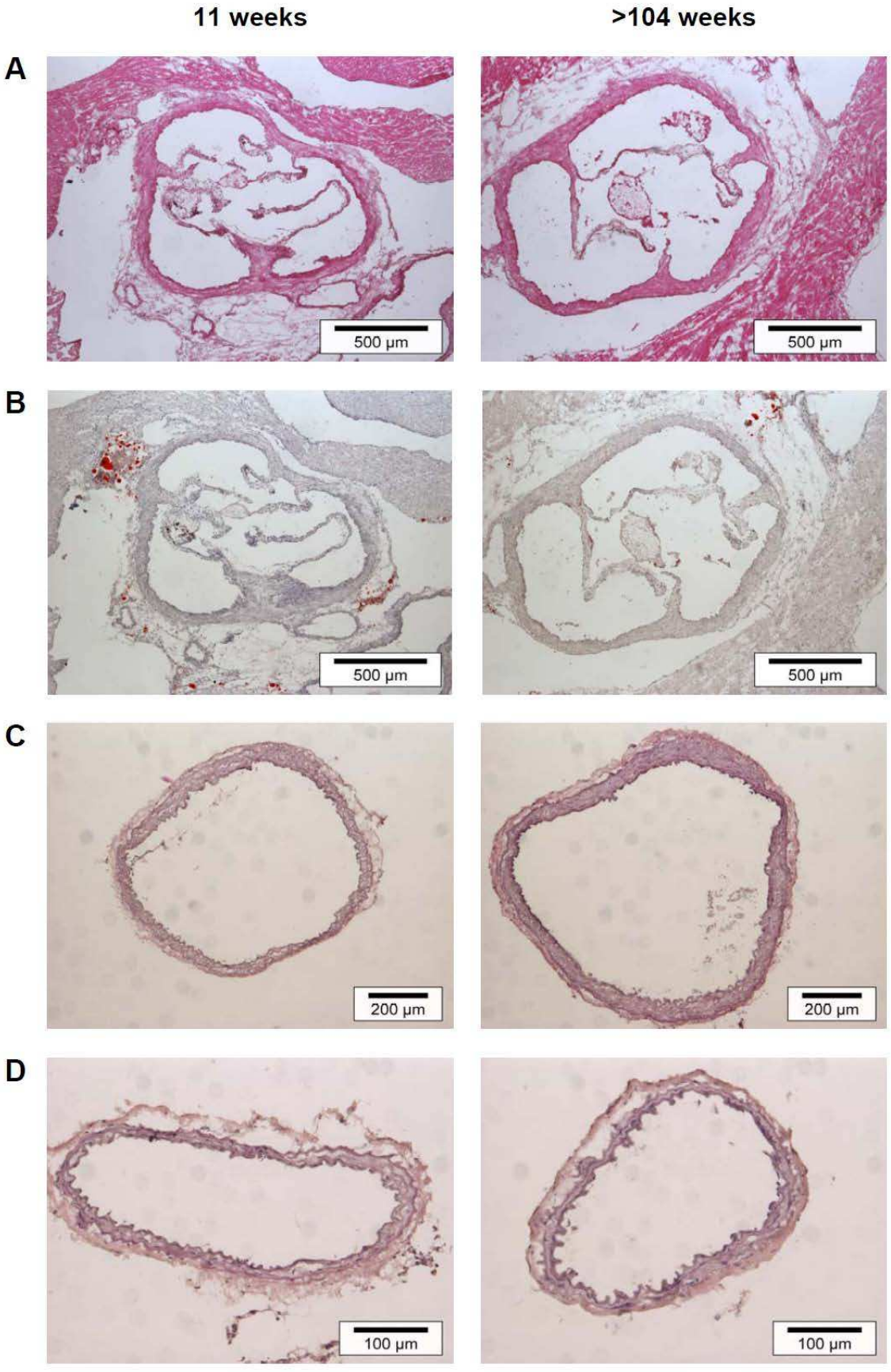
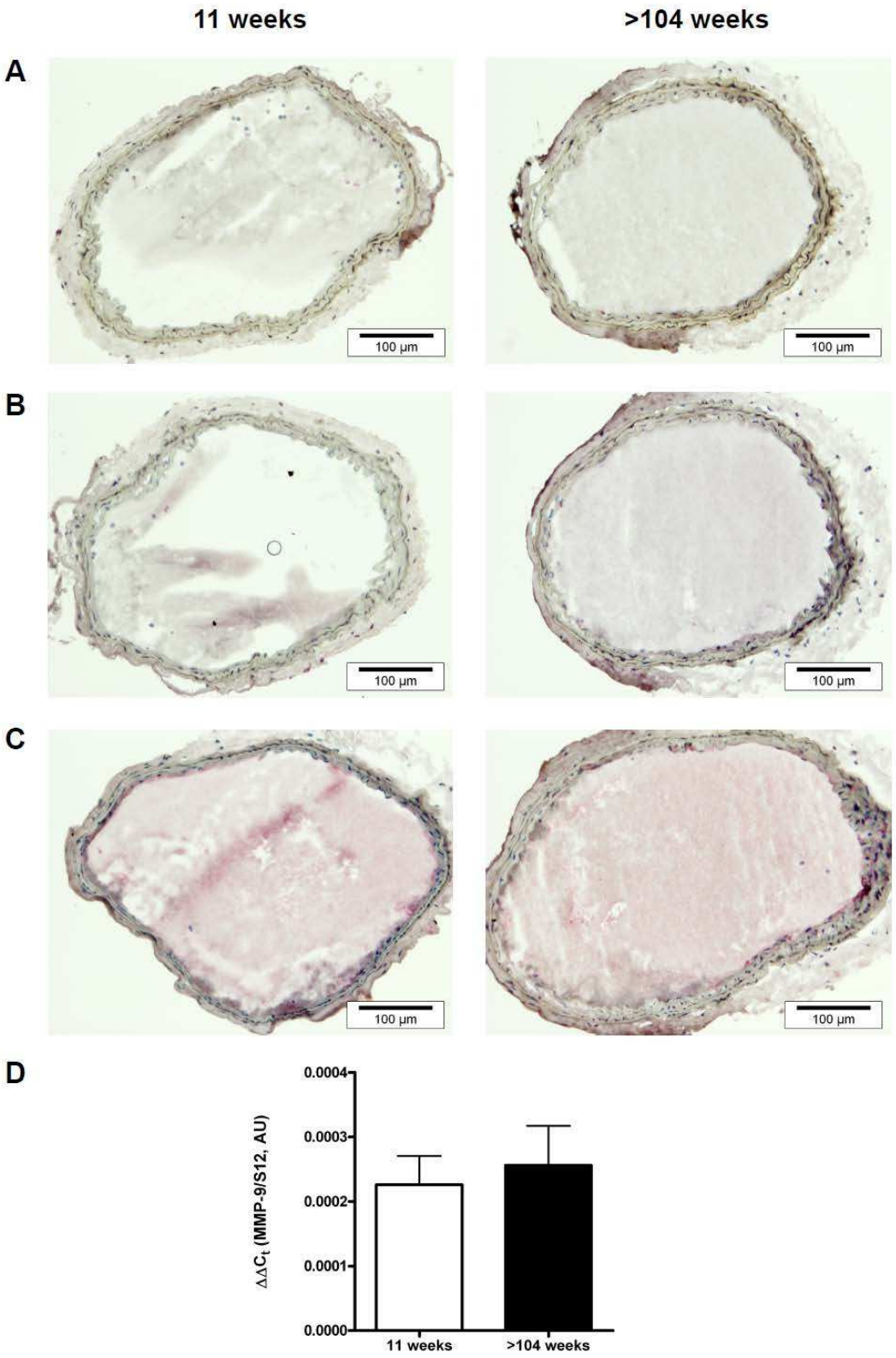

11 weeks
>104 weeks
A
500 µm
500 µm
B
500 µm
500 µm
C
200 µm
200 µm
D
100 µm
100 µm

Figure 5


11 weeks
>104 weeks
A
B
C
D
100 µm
100 µm
100 µm
100 µm
100 µm
100 µm
ΔΔCt (MMP-9/S12, AU)
0.0004
0.0003
0.0002
0.0001
0.0000
11 weeks
>104 weeks